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Clinical Evaluation of Tumour-Infiltrating Lymphocytes as a Prognostic Factor in Human Papillomavirus-Associated Oropharyngeal Squamous Cell Carcinoma

Running title:

TILs in HPV-Associated Oropharyngeal Carcinoma

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The authors have no conflict of interest to declare

Abstract

Background

The majority of patients with human papillomavirus (HPV)-associated oropharyngeal squamous cell carcinoma (OpSCC) have favourable survival outcomes, but a significant minority of individuals will die of their disease. There are currently no definitive criteria to identify HPV-associated OpSCC patients with poor outcome. Recent reports suggest that quantitative evaluation of T-cell subpopulations in OpSCC may be of prognostic value, but the methods used have limited utility in a clinical diagnostic setting. We sought therefore to determine the clinical prognostic utility of tumour-infiltrating lymphocyte (TIL) evaluation in HPV-associated OpSCC within the context of a diagnostic histopathology setting.

Methods

Representative diagnostic haematoxylin and eosin (H&E) stained slides from 232 consecutive HPV-associated OpSCC patients were classified as containing a high (TIL_{Hi}; diffuse, lymphocytes in >80% of tumour and stroma), moderate (TIL_{Mod}; patchy, present in 20-80% of tumour and stroma) or low (TIL_{Lo}; sparse or absent, present in <20% of tumour and stroma). Inter-observer reliability was assessed, and TILs category was then correlated with overall survival (OS) and disease-free survival (DFS).

Results

Univariate and multivariate analysis showed a statistically significant difference in overall and disease-free survival estimates when TIL_{Hi} and TIL_{Mod} groups were compared with TIL_{Lo} patients (p<0.0001 TIL_{Hi} vs TIL_{Lo}, p<0.0001 TIL_{Mod} vs TIL_{Lo}). Statistical significance was retained when TIL_{Hi} and TIL_{Mod} were grouped into a single category (TIL_{Hi}) and compared to TIL_{Lo} (p<0.0001).

Conclusion

We demonstrate the prognostic utility of TILs in HPV-associated OpSCC in clinical practice. A binary system classifying HPV-associated OpSCC into TIL_{Hi} and TIL_{Lo} on routine H&E staining stratifies patients into potentially favourable and unfavourable survival outcomes, respectively.

Keywords:

Tumour infiltrating lymphocytes; TIL; TILs; Human papillomavirus; HPV; Oropharyngeal carcinoma

Background

The dramatic rise in the incidence of oropharyngeal squamous cell carcinoma (OpSCC) in Europe and North America in the past two decades is attributable to high-risk types of human papillomavirus (HPV). In parallel with this striking epidemiological trend is the observation that patients with HPV-associated OpSCC have significantly improved overall- and disease-free survival outcomes compared to site- and stage-matched HPV-negative tumours[1]. While the majority of patients with HPV-associated OpSCC have favourable survival, a significant minority of these patients experience treatment resistance resulting in poor outcomes[2, 3].

Treatment failure in HPV-associated OpSCC is likely to be multifactorial, but emerging data suggest that the tumour immune microenvironment is likely to influence clinical outcomes. Recently, several studies utilising lymphocyte subpopulation-specific detection techniques have demonstrated a positive correlation between tumour-infiltrating lymphocytes (TILs) and improved survival in HPV-associated OpSCC [4-9]. Within the context of a clinical histopathology setting from a single institution cohort, we sought to retrospectively validate the prognostic utility of TILs in HPV-associated OpSCC using diagnostic haematoxylin and eosin (H&E) stained sections.

Materials and methods

Consecutive patients diagnosed with HPV-associated OpSCC in the Department of Head and Neck Pathology, Guys' & St Thomas' NHS Foundations Trust between January 2005 and December 2017 were retrospectively identified from pathology

databases by members of the responsible clinical team, in compliance with the UK Data Protection Act. HPV testing was undertaken at the time of diagnosis according to current guidelines[10]. p16 immunohistochemistry (clone E6H4, CINtec, Roche, UK) was performed on an automated platform (Benchmark Ultra, Ventana Medical Systems, USA) according to manufacturer's instruction as previously described[11]. OpSCCs demonstrating strong and diffuse nuclear and cytoplasmic positivity in >70% of tumour cells were then subject to high-risk HPV testing by DNA in-situ hybridisation (INFORM Family III, Roche, UK) according to manufacturer's instruction as previously described[11]. Only OpSCCs demonstrating positivity for both p16 immunohistochemistry and high-risk HPV DNA by in-situ hybridisation were included in this study. TILs were evaluated on at least one representative whole-mount diagnostic H&E slide from the primary tumour. Two pathologists (KR and ST) independently scored TILs according to a ternary classification system as described by Ward and colleagues[6]. Lymphocytes present within tumour nests/sheets, in the stromal component between tumour nests and in the normal lymphoid component of the tonsil and base of tongue were included. Any lymphocytes beyond the tumour invasive front, plasma cells and neutrophils were excluded from TILs assessment. Whole-section area was then categorised as high (TIL_{Hi}; diffuse, present in >80% of tumour and stroma), moderate (TIL_{Mod}; patchy, present in 20-80% of tumour and stroma) or low (TIL_{Lo}; sparse or absent, present in <20% of tumour and stroma). We then also reclassified all tumours using a binary system as TIL_{Hi} (diffuse or patchy, present in >20% of tumour and stroma) or TIL_{Lo} (sparse or absent, present in <20% of tumour and stroma). Representative photomicrographs of TIL_{Hi} and TIL_{Lo} are demonstrated in Figure 1. Discordant categorisation was resolved in a consensus meeting between three pathologists (KR, AS and ST). Patient demographics, overall

survival (OS) and disease-free survival (DFS) were obtained from patient records and anonymised. Inter-rater agreement and survival estimate analyses were calculated using SPSS for Windows version 25 (IBM, Portsmouth, UK). Kappa and weighted kappa statistics, Kaplan-Meier log rank and Cox proportional hazard regression analyses were used to evaluate inter-rater reliability, univariate survival and multivariate survival, respectively.

Results

Slides of incisional biopsies or diagnostic tonsillectomies from 232 retrospectively identified primary HPV-associated OpSCCs were available for TILs evaluation. There were 188 males and 44 females (M:F=4.3:1) with a mean age of 59 years (range 35-83 years). Staging data were available for 224 patients and were as follows: T1=52, T2=87, T3=30, T4=55; N0=24, N1=19, N2a=34, N2b=90, N2c=43, N3=14; M0=216, M1=8). All patients received primary radiotherapy or chemoradiotherapy.

The inter-rater kappa scores using the ternary and binary systems were 0.72 (weighted) and 0.87, respectively. Following consensus classification using the ternary system, there were 104 (44.8%), 97 (41.8%) and 31 (13.4%) tumours categorised as TIL_{Hi}, TIL_{Mod} and TIL_{Lo}, respectively. Using the binary system, there were 201 (86.6%) TIL_{Hi} and 31 (13.4%) TIL_{Lo} tumours.

Using the ternary system, the mean OS estimates were 55.8 (95%CI: 53.2-58.4), 52.1 (95%CI:48.5-55.7) and 37.4 (95%CI:28.8-46.0) months for TIL_{Hi}, TIL_{Mod} and TIL_{Lo}, respectively. There was a statistically significant difference in OS estimates

when TIL_{Hi} and TIL_{Mod} groups were compared with TIL_{Lo} patients (p<0.0001 TIL_{Hi} vs TIL_{Lo}, p<0.0001 TIL_{Mod} vs TIL_{Lo}). However, differences in OS estimates did not reach statistical significance when TIL_{Hi} and TIL_{Mod} groups were compared (p=0.071, Figure 2A). Mean DFS estimates were 58.8 (95%CI: 57.5-60.2), 53.8 (95%CI:50.4-57.2) and 44.8 (95%CI:35.9-53.7) months for TIL_{Hi}, TIL_{Mod} and TIL_{Lo}, respectively. There was a statistically significant difference in DFS estimates between all three groups (p<0.0001 TIL_{Hi} vs TIL_{Lo}, p=0.025 TIL_{Mod} vs TIL_{Lo}, p=0.008 TIL_{Hi} vs TIL_{Mod} Log Rank, Figure 2B). After adjusting for age, gender, T, N and M, there was a significant difference (p=0.006) between TIL_{Mod} and TIL_{Lo} for OS. Those with TIL_{Lo} were 3.41 times more likely to not survive compared to those with TIL_{Mod} (Hazard Ratio 3.41, 95%CI:1.42-8.15). There was no significant difference between TIL_{Hi} and TIL_{Mod} (p=0.129). For DFS, there was a significant difference (p=0.003) between TIL_{Lo} and TIL_{Mod}. Those with TIL_{Lo} were 5.21 times more likely to not survive compared to those with TIL_{Mod} (Hazard Ratio 5.21, 95%CI:1.74-15.62). There was no significant difference between TIL_{Hi} and TIL_{Mod} (p=0.154).

Using the binary system, mean OS estimates were 54.1 (95%CI:51.8-56.3) and 37.4 (95%CI:28.8-46.0) months for TIL_{Hi} and TIL_{Lo}, respectively (p<0.0001, Log Rank; Figure 2C). Mean DFS estimates were 56.4 (95%CI:54.6-58.2) and 44.4 (95%CI:35.9-53.7) months for TIL_{Hi} and TIL_{Lo}, respectively p<0.0001, Figure 2D). After adjusting for age, gender, T, N and M there was a significant difference (p<0.0001) between TIL_{Hi} and TIL_{Lo} for OS. Those with TIL_{Hi} were 4.55 times more likely to survive compared to those with TIL_{Lo} (Hazard Ratio 0.22, 95%CI:0.10-0.51). For DFS, there was a significant difference (p<0.0001) between TIL_{Hi} and TIL_{Lo} for DFS. Those with TIL_{Hi} were 7.14 times more likely to survive compared to those with TIL_{Lo} (Hazard Ratio 0.14, 95%CI:0.05-0.42).

Discussion

Calls for the introduction of treatment de-intensification regimens in HPV-associated OpSCC is founded on robust evidence that patients with this disease have improved overall- and disease-specific survival compared to site and stage-matched individuals. Despite this observation, approximately 20% of patients with HPV-associated OpSCC are estimated to die of their disease and for whom treatment de-intensification would be inappropriate[3]. There is therefore a need to refine inclusion criteria in de-intensification clinical trials beyond HPV status alone.

Against this background, emerging evidence from several groups utilising quantitative and semi-quantitative methods for evaluating T-cell subpopulations raise the possibility that TILs may act as a potential prognostic biomarker in HPV-associated OpSCC[5-9, 12]. Interestingly, Ward et al demonstrated that for purposes of prognostication, subjective evaluation of TILs on H&E stained sections performed equally well as more complex quantification of CD3, CD4, CD8, and FoxP3 T cell subpopulations[6]. In accordance with the work of Ward et al., our study also showed that TILs assessment on H&E sections has significant prognostic value and therefore overcomes the need for further ancillary testing. Within the context of routine clinical diagnostic practice, less complex prognostic tests are more likely to be adopted in view of cost and turnaround time benefits.

Unlike the study by Ward et al, there was no statistically significant difference in overall survival between the TIL_{Hi} and TIL_{Mod} in our study cohort, an observation which may be explained, at least in part, by the subjective nature of TILs scoring. In our study, a statistically significant difference ($p<0.0001$) in overall survival remained

when TIL_{Hi} and TIL_{Mod} were group together and compared with TIL_{Lo}. Binary classification systems are likely to be of greater clinical utility compared to ternary scoring systems since treatment protocols are more difficult to define in any 'intermediate' group.

Inter-observer reproducibility is another factor key to the adoption of any histological biomarker into clinical practice. In the current study, there was a good inter-rater weighted kappa value of 0.72 using the ternary scoring system. Unsurprisingly, the inter-rater kappa score improved (0.87) when $TIL_{Hi\ and}$ TIL_{Mod} were grouped and compared to TIL_{Lo}. Since a binary TILs scoring system results in greater inter-rater correlation without compromising prognostic performance, we recommend that HPV-associated OpSCC should be classified as TIL_{Hi} or TIL_{Lo} using a cut-off value of 20% tumour and stroma containing the presence of lymphocytes.

In summary, we demonstrate the prognostic utility of TILs in HPV-associated OpSCC in clinical practice using a binary system where the presence of lymphocytes in 20% of tumour and stroma acts as a cut-off to classify HPV-associated OpSCC into high and low TILs groups, respectively. Further work, including standardisation of scoring criteria, validation in different population cohorts and prospective evaluation of recommended cut-offs are necessary prior to implementation of TILs as a criterion for treatment selection in de-intensification regimens.

Author contribution

KR, AS, ST: Concept, methodological design, data acquisition and manuscript preparation.

FW: Methodological design and statistical analysis.

TGU, MRF: Concept, manuscript preparation.

ML: Concept, data acquisition.

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Figure legends

Figure 1. Representative photomicrographs of TIL_{Lo} (**A** low power magnification, B medium power magnification) and TIL_{Hi} (**C** low power magnification, **D** medium power magnification, H&E) HPV-associated OpSCCs.

Figure 2. Kaplan-Meier overall (**A**, **B**) and disease-free (**C**, **D**) survival plots of HPV-associated OpSCC the ternary (**A**, **C**) and binary (**B**, **D**) TILs classification system.